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CD151 regulates expression of FGFR2 in breast cancer cells via PKC-dependent pathways.

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Summary statement

We demonstrate that cancer related tetraspanin protein CD151 uses novel pathways in the regulation of expression of fibroblast growth factor receptor 2 (FGFR2).

Abstract

Expression of the tetraspanin CD151 is frequently upregulated in epithelial malignancies and correlates with poor prognosis. Here we report that CD151 is involved in regulation of the expression of fibroblast growth factor receptor 2 (FGFR2). Depletion of CD151 in breast cancer cells resulted in an increased level of FGFR2. Accordingly, an inverse correlation between CD151 and FGFR2 was observed in breast cancer tissues. CD151-dependent regulation of the FGFR2 expression relies on post-transcriptional mechanisms involving HuR/ELAVL1, a multifunctional RNA binding protein, and the assembly of processing bodies (P-bodies). Depletion of CD151 correlated with inhibition of PKC, a well-established downstream target of CD151. Accordingly, the levels of dialcylglycerol species were decreased in CD151-negative cells, and inhibition of PKC resulted in the increased expression of FGFR2. Whilst expression of FGFR2 itself did not correlate with any of the clinicopathological data, the FGFR2-/CD151+ patients are more likely to develop lymph node metastasis. Conversely, FGFR2-/CD151- patients demonstrated better overall survival. These results illustrate functional interdependency between CD151 complexes and FGFR2 and suggest a previously unsuspected role of CD151 in breast tumourigenesis.

Key words: CD151, FGFR2, breast cancer, tetraspanin, PKC

Introduction

Tetraspanins represent a large group of four transmembrane domain proteins with diverse biological activities (Berditchevski and Rubinstein, 2013). At the biochemical level tetraspanins are thought to function as the main structural blocks and organisers of distinct microdomains on the plasma membrane which also include transmembrane receptors (e.g. integrins, receptor tyrosine kinases) and cytoplasmic signalling proteins. Analyses of clinical material have suggested that several tetraspanin proteins may be involved in the development and metastatic progression in various cancer types (Hemler, 2014).

Expression of the tetraspanin CD151 is elevated in various types of breast cancer and this correlated with poor prognosis and overall survival in breast cancer patients (Kwon et al., 2012; Novitskaya et al., 2014). Experiments involving cell lines and animals models suggested that protumourigenic and pro-metastatic functions of CD151 are likely to be dependent on its ability to form complexes with laminin-binding integrin receptors (i.e. α6β1, α3β1 and α6β4) and coordinate integrin-dependent signalling networks in the context of receptor tyrosine kinases (Sadej et al., 2014). Specifically, CD151 is known to regulate integrin ligand binding and post-adhesion signalling including activation of small GTPases (Rho, Rac, cdc42), FAK, cAkt and Erk1/2 (Sadej et al., 2014). The association with integrins is also important for CD151--dependent regulation of cellular responses to growth factors and inhibitory drugs that target receptor tyrosine kinases (RTKs) in cancer cells (Deng et al., 2012; Novitskaya et al., 2014; Sadej et al., 2010). Cross-talk between integrins and RTKs was shown to involve classical protein kinase C (PKC), well-established molecular partners for several tetraspanin proteins including CD151 (Li et al., 2013). In addition to regulating integrin function, CD151 may influence the metastatic potential of cancer cells indirectly via E-cadherin–based cell-cell adhesion complexes (Johnson et al., 2009; Shigeta et al., 2003), or through the increased production of extracellular matrix degrading enzymes (e.g. MMPs) (Hasegawa et al., 2007).

FGFR2 is a member of a receptor tyrosine kinase subfamily which also includes FGFR1, FGFR3 and FGFR4 (Kelleher et al., 2013). There is increasing evidence that the FGF-FGFR2 signalling axis plays an important role in breast cancer. Genome wide analysis has identified a number of single nucleotide polymorphic (SNP) variants in intron 2 of the FGFR2 gene which were associated with a higher incidence of risk of breast cancer (Cui et al., 2016), and in particular, in patients positive for hormonal receptors and negative for Her2/ErbB2 (Cox et al., 2016). Several transcription factors (e.g. FoxA1, Oct1, E2F1) were shown to bind differentially to the high-risk alleles and, therefore, might be responsible for elevated expression of FGFR2 in patients carrying these alleles (Robbez-Masson et al., 2013). Furthermore, gene amplification and overexpression of FGFR2 in breast cancer tissues has been also described, particularly in the triple negative (i.e. ER-/PR-/ErbB2-negative) breast cancers (Turner et al., 2010). Accordingly, overexpression and FGFR2 inhibitor studies using human cell models supported the pro--tumourigenic function of FGFR2 in breast cancer (Bai et al., 2010; Sommer et al., 2016). On the other hand, activation of FGFR2 in mouse primary mammary epithelial cells resulted in activation of apoptosis (Xian et al., 2007). It was also reported that FGFR2 inhibited epithelial-to-mesenchymal transition and attenuated growth of human breast cancer xenografts *in vivo* (Tarkkonen et al., 2012).

Here we describe a new link between tetraspanin CD151 and FGFR2 in breast cancer. Specifically, we found that CD151 controls the expression level of FGFR2 using a pathway which is independent of proteolytic degradation or transcriptional regulation. Instead, it involves the assembly of processing bodies (P-bodies) and PKC-dependent signalling pathways. Importantly, the inverse correlation between expression of CD151 and FGFR2 in cellular models of breast cancer was also observed in breast cancer tissues thus emphasizing the importance of our findings for future translational studies.

RESULTS

CD151 regulates expression of FGFR2 gene. While analysing the expression of FGF receptors and tetraspanin proteins in a panel of breast cancer cell lines, we noticed a tendency for an inverse correlation between the levels of FGFR2 and CD151 (Fig. 1A). In these experiments we detected no correlation between expression levels of CD151 and FGFR1 on one hand, or between FGFR2 and several other tetraspanins (i.e. CD9, CD63, CD81 and CD82), on the other (Fig.1A and results are not shown). In agreement with this observation stable silencing of CD151 (but not CD63 or CD81) resulted in the increased FGFR2 levels in HB2, a DCIS-like mammary epithelial cell line, as well as in SKBR3 and MCF7 – two widely used breast cancer cell models (Fig. 1B and results are not shown). The re-expression of CD151 in HB2/CD151(-) cells reverted the FGFR2 protein level to that observed in HB2/CD151(+) cells, thus further excluding the off-target effect of the shRNACD151 construct (Fig.1C). An increased level of FGFR2 was also observed when CD151 was targeted with an alternative siRNA in transient knockdown experiments (Supplementary Fig. 1). Importantly, knockdown of CD151 had no effect on expression levels of FGFR1 or FGFR4 (FGFR3 was not detectable), emphasizing the specific relationship between CD151 and FGFR2 (Fig. 1B).

Previous studies have demonstrated that most of CD151-dependent regulation of cellular function involves laminin-binding integrins ($\alpha3\beta1$ and $\alpha6$ integrins) (Sadej et al., 2014). Surprisingly, we found that depletion of these integrins in HB2 cells, either separately or in combination, had no effect on the expression level of FGFR2 (Fig. 1D), thus demonstrating that the CD151-dependent regulation of the FGFR2 expression does not involve integrins.

Depletion of CD151 accentuates responses of mammary epithelial cells to FGFs. The initial proliferation experiments demonstrated that HB2/CD151(+) and HB2/CD151(-) cells responded similarly to FGF2 and FGF9 when cultured under standard 2D conditions (Supplementary Fig.2 and results are not shown). In contrast, the growth response of CD151-depleted

cells to FGFs was more robust when cells were placed in 3D-lrECM (Fig. 2A). Furthermore, we noticed that FGF-treated HB2/CD151(-) colonies lost their smooth, “ball-like” morphology and appeared as disorganized aggregates of cells (Fig. 2A). Importantly, re-expression of the wild type CD151 reversed proliferative and morphological phenotypes of HB2/CD151(-) cells (Fig. 2B). Similarly, FGF-induced proliferation of CD151–depleted SKBR3 cells in 3D-lrECM was more pronounced when compared to the control, SKBR3/CD151(+) cells (Fig. 2C). Analysis of FGF-triggered signalling of HB2 cells growing in 3D-lrECM revealed higher levels of phosphorylation of FRS2 and PLCγ (specific downstream effectors of FGFR-triggered signalling) in CD151-depleted cells (Fig. 2D). Taken together these results demonstrate that elevated FGFR2 levels in CD151-depleted cells translate into more prominent responses of breast cancer cells to FGFs.

CD151 regulates expression of FGFR2 at the post-transcriptional level. To investigate how CD151 controls the expression of FGFR2 we have initially examined the effect of inhibitors of various proteolytic pathways. In these experiments we found that treatment of HB2 cells with inhibitors of matrix metalloproteases (GM6001, Batimastat), serine proteases (leupeptin), aspartic proteases (pepstatin), calpains (calpastatin) and caspases (Z-VAD-FMK) did not change expression levels of FGFR2 either in CD151-positive or CD151-negative cells (Supplementary Fig.3 and not shown). Likewise, bafilomycin A1 and MG132, general inhibitors of endosomal/lysosomal and proteosomal degradation have no notable effect on FGFR2 levels in HB2/CD151(+) and HB2/CD151(-) cells (Supplementary Fig.3 and not shown). Therefore, we concluded that CD151-dependent regulation of the FGFR2 expression does not involve common proteolytic pathways.

In further experiments we compared the levels of FGFR2 mRNA in the control and CD151-depleted cells. Several probes covering various parts of FGFR2 mRNA were used in quantitative RT-PCR experiments to account for possible variations in FGFR2 splicing. There were no differences observed between the control and CD151-depleted cells in these experiments (Supplementary Fig.4), thus ruling out the regulation at the level of mRNA.

Transcribed mRNAs bind a diverse range of nuclear proteins which facilitate their export to the cytoplasm where the mRNP complexes are either immediately translated or stored in various types of cytoplasmic granules (Buchan, 2014). Thus, we examined whether depletion of CD151 affected the assembly of stress granules (SG), P-bodies and GW bodies, three most common types of RNA-containing granules in eukaryotic cells. In these experiments we found that the number of P-bodies was noticeably reduced in CD151-negative HB2 and SKBr3 cells (Fig. 3A). By contrast, distribution of TIA-1 (SG marker) and GW182 (a marker for GW bodies) was comparable in HB2/CD151(+) and HB2/CD151(-) (results are not shown). To examine the role of P-bodies in FGFR2 expression directly, we have silenced the expression of EDC4 and Pat1b, which were shown to regulate the assembly of P-bodies. As illustrated in Figure 3B, depletion of either proteins increased FGFR2 levels. Therefore, we concluded that the elevated expression of FGFR2 in CD151-negative cells is likely to involve P-bodies.

The mRNA binding protein ELAVL-1/HuR is involved in regulation of FGFR2 expression. Nuclear/cytoplasmic proteins TIA-1, TIAR, hnRNP-C1/C2 and ELAVL-1/HuR were shown to bind FGFR2 mRNA and known to be functionally linked to P-bodies. Therefore, we examined whether these proteins can be involved in CD151-dependent regulation of expression of FGFR2 protein in breast cancer cells. Total expression levels and nuclear vs cytoplasmic accumulation of these proteins were comparable in CD151-positive and CD151-negative cells (Fig. 4A). Knockdown of ELAVL-1/HuR resulted in the increased level of FGFR2 protein in HB2 and SKBr3 cells (Fig. 4B). By contrast, the expression level of FGFR1 was not affected (Supplementary Fig.5A). Furthermore, depletion of TIA-1, TIAR or hnRNP-C1/C2 had no or a minimal effect on the expression levels of FGFR2 in either CD151-positive or CD151-negative cells (Supplementary Fig.5B).

CD151-dependent regulation of FGFR2 expression involves PKC. To examine molecular mechanisms that could link CD151 with the activity of ELAVL-1/HuR towards FGFR2, we analysed signalling pathways that are known to be regulated by tetraspanins, on one hand, and affect the function of

ELAVL-1/HuR, on the other. Specifically, we compared activation of Src, p38 and PKC in the control and CD151-depleted HB2 cells. As illustrated in Figure 5A the levels of active Src and p38 kinases were comparable in HB2/CD151(+) and HB2/CD151(-) cells, thereby ruling out the involvement of these proteins in CD151-dependent regulation of FGFR2 expression. CD151 was shown to associate with classical protein kinases C (cPKC) (Zhang et al., 2001). Interestingly, we found the phosphorylation levels of several cellular proteins recognized by the phospho-(Ser) PKC substrate antibody were higher in CD151-positive HB2 cells (Fig. 5B) thus indicating that depletion of CD151 resulted in the lower basal level of activation of PKC. Importantly, treatment of cells with Bisindolylmaleimide I (BIM I), a highly specific PKC inhibitor, increased FGFR2 levels in both HB2 and SKBr3 cells (Fig. 5C). Interestingly, treatment of HB2/CD151(+) cells with BIM I had no effect on the assembly of P-bodies (Supplementary Fig.6). These results suggest that although activation of PKC is important for regulation of FGFR2 expression by CD151, other pathways are responsible for the effect of CD151 on the assembly of P-bodies.

It has been previously reported that when activated by PMA/TPA classical PKC form complexes with various tetraspanin proteins including CD151 (Termini and Gillette, 2017). Thus, increased activation of PKC in CD151-positive breast cancer cells might have been due to the association with tetraspanin microdomains (or, more specifically, with CD151-containing complexes). However, in accordance to previous studies, PKC was not a part of the CD151-complexes purified from cells grown under standard culturing conditions (Supplementary Fig.7). We therefore considered whether increased PKC activation in CD151-positive cells is due to differences in the levels of diacylglycerol (DAG), the physiological activator of classical and novel PKC. The quantitative analysis of DAG species in HB2/CD151(+) and HB2/CD151(-) cells has revealed that depletion of CD151 resulted in 1.5-2 fold decrease in the levels of various DAGs species with the most pronounced differences observed for 34:1, 36:1, 36:2 and 38:4 species (Fig.5D). Importantly, the effect of CD151 depletion on these DAGs was specific as we observed no differences in the levels of some other species (e.g. 32:1 and

34:2). Although, differences in the levels of DAGs were not due to the activity of PLCγ (as monitored by the phosphorylation levels of Tyr783) (Fig.5E), we found that depletion of CD151 has resulted in changes in the intracellular distribution of the enzyme with most cells losing its prominent PLCγ-positive punca/vesicles (Fig.5F). Thus, we concluded, that CD151-dependent regulation of PKC activation and, subsequently, expression of FGFR2 is controlled by the abundance of particular DAGs which may be caused by changes in compartmentalization of PLCγ.

Inverse relationship between FGFR2 and CD151 but not FGFR2 alone is associated with invasiveness of breast cancer. To examine links between FGFR2 and CD151 further, we analysed coexpression of these proteins in tissue samples from patients with invasive ductal carcinoma (IDC) (Table 1). In the majority of both normal mammary glands and tumours, expression of FGFR2 was highly heterogeneous. Various patterns of FGFR2 immunostaining, i.e. cytoplasmic/membranous (39%; 65/166), nuclear (45%; 75/166) or ubiquitous (23%; 38/166) could be seen. There was no association between levels of FGFR2 immunoreactivity in distinct cellular compartments. A highly heterogeneous pattern of immunoreactivity was also observed when expression of FGFR2 was analysed in combination with immunoreactivity for CD151. Inverse FGFR2/CD151 relationship was seen in 51/166 (31%) and 38/166 (23%) cases for FGFR2(-)/CD151(+) and FGFR2(+)/CD151(-), respectively. In 50/166 cases (30%) tumours were negative for both FGFR2 and CD151, while a FGFR2(+)/CD151(+) trait was found in minority of samples (27/166; 16%). Importantly, Kendall's test demonstrated a nonlinear negative correlation between cytoplasmic/membranous FGFR2 and CD151 (τ=0.14, p=0.029), thus supporting the inverse relationship between FGFR2 and CD151 levels in breast cancer cells. Expression of nuclear FGFR2 alone did not correlate with any of the tumour characteristics and cytoplasmic/membranous FGFR2 was significantly associated only with lack of hormone receptors (p=0.04) (Table 1). However, when assessed in combination with CD151, an inverse FGFR2/CD151 expression in tumour cells was significantly correlated with clinicopathological variables such as grade (FGFR2(+)/CD151(-) vs. rest; p=0.012) and nodal status (FGFR2(-

)/CD151(+) vs rest; p=0.037) (Tables 1 and 2). FGFR2(-)/CD151(+) phenotype was also more frequently seen in the triple-negative subgroup of the studied cohort (p=0.039) (Table 1). Expression of FGFR2 alone (either cytoplasmic/membranous or nuclear) was of no prognostic value (Fig. 6A). Interestingly, its absence from the cell, when combined with lack of CD151 expression (FGFR2(-)/CD151(-)), not only inversely correlated with nodal status (p=0.013) and HER2 (p=0.014) (Tables 1 and 2), but also proved a good prognostic indicator (p=0.024) (Fig. 6B), albeit not independent (results of multivariate analysis not shown).

Discussion

Dysregulation of the FGF-FGFR2 signalling network is thought to play an important role in various epithelial malignancies (Katoh and Nakagama, 2014). Point mutations, gene amplification, tumour associated alternative splicing and gene rearrangements can lead to changes in FGFR2 functions (Fearon et al., 2013; Katoh, 2008; Katoh, 2009; Wu et al., 2013). Here we show for the first time that the expression of FGFR2 in breast cancer cells is controlled by the CD151-based signalling complexes. Importantly, we established that CD151-dependent post-transcription regulation of FGFR2 expression is specific (i.e. CD151 does not regulate expression of FGFR1 or FGFR4) and involves PKC.

Importantly, in agreement with our *in vitro* observation, an inverse correlation between expression of CD151 and FGFR2 was also seen in the tumour tissues. These results imply that the pathway(s) which links expression of these proteins is(are) also operational *in vivo*. Expression of FGFR2 alone did not have any prognostic value, which confirms our recent observations (Czaplinska et al., 2016). Although hormone receptors were the only clinicopathological feature associated with FGFR2, in contrast to the previous study, its relationship was inverse. The discrepancy between these results may reflect differential roles played by FGFR2 in distinct subtypes of breast carcinoma, as unlike findings shown by Czaplinska and colleagues, our analysis was done on a histologically homogeneous group of specimens (i.e. invasive ductal carcinoma). In the patients with inverse correlation of CD151

and FGFR2 expression, only those that expressed high levels of CD151 (i.e. FGFR2(-)/CD151(+)) demonstrated correlation with lymph node involvement. Interestingly, a cohort of FGFR2(+)/CD151(+) patients did not show such correlation, which suggests that the presence of FGFR2 may suppress prometastatic activity of CD151. Indeed, this and previous studies (Klosek et al., 2009; Kwon et al., 2012) revealed a highly significant correlation between the expression of CD151 and metastasis to the lymph nodes. Conversely, only in the absence of FGFR2 expression, CD151-negative cancer cells (FGFR2(-)/CD151(-) cohort) were less likely to be found in the lymph nodes. These results suggest that the proposed prometastatic function of CD151 in breast and, possibly, other cancers should be assessed in the context of FGFR2 expression.

We discovered that CD151-dependent regulation of FGFR2 expression occurs at the posttranscriptional level via the mechanisms involving P-bodies. P-bodies are ribonucleoprotein containing cytoplasmic aggregates which are known to control mRNA stability and translation (Anderson et al., 2015). The involvement of P-bodies adds a new layer of complexity to regulation of FGFR2 expression. Our data strongly suggest that HuR/ELAVL-1 is likely to be a critical downstream component of the CD151-dependent signalling network which is targeting FGFR2 mRNA. In other experiments we found that FGFR2 is not the only target for HuR/ELAVL-1 in HB2 cells: expression levels of both c-myc and cyclin E, two previously identified targets for HuR/ELAVL-1 were also increased in HB2/CD151(-) cells (Supplementary Fig.8). These results suggest that CD151-dependent regulation of the HuR/ELAVL-1 function may have wider consequences for CD151-positive breast cancer cells.

HuR/ELAVL-1 is a widely expressed multifunctional RNA binding protein which was shown to regulate alternative splicing, mRNA stability and translation by binding to AU-rich elements (AREs) in the 3'- and 5'-untranslated regions of multiple mRNAs (Lebedeva et al., 2011; Uren et al., 2011). Whilst specific involvement of HuR/ELAVL-1 in regulation of FGFR2 expression has not been reported previously, the mFGFR2 was identified in the transcriptome-wide screen for potential HuR/ELAVL-1 targets (Lebedeva

et al., 2011). Furthermore, the recent report by Hubstenberger and colleagues demonstrated that HuR/ELAVL-1 and mRNA for FGFR2 segregate to P-bodies (Hubstenberger et al., 2017). Alternatively, HuR/ELAVL-1 can control the expression and/or alternative splicing of various proteins whose function is directly linked to the P-body assembly (Lebedeva et al., 2011; Uren et al., 2011).

RNA-binding and nuclei-cytoplasm shuttling of HuR/ELAVL-1 are controlled by various post-translational modifications of the protein and involve a multitude of signalling pathways (Eberhardt et al., 2012; Grammatikakis et al., 2017). Our data suggest that CD151 regulates the activity of the protein via a PKC-dependent signaling pathway. A functional link between CD151 and cPKC has been demonstrated in two earlier studies (Li et al., 2013; Shigeta et al., 2003). Although not directly examined, both reports suggested that the expression of CD151 is correlated with the increased activity of cPKC towards their substrates. Using antibodies that detect phosphorylation of PKC substrates as our biochemical readout, we demonstrated that the activity of PKC is, in fact, suppressed in CD151-depleted cells. This poses an important question regarding the nature of the cPKC-CD151 interaction and how the removal of the tetraspanin can affect the activity of the enzymes. Previous biochemical studies have shown that the interaction between tetraspanins and cPKC occurs only under the acute cell stimulation with TPA/PMA (Gustafson-Wagner and Stipp, 2013; Zhang et al., 2001) or upon activation EGF-/B-cell receptors (Deng et al., 2012; Zuidscherwoude et al., 2017). Here we demonstrated for the first time that a tetraspanin protein affects PKC-dependent signalling even under standard/basal growth conditions when the association between the proteins is not detected (Suppl Fig.5). This observation indicates that the physical link between CD151 and cPKC is not necessary for the tetraspanin to modulate the activity of the enzymes towards their targets. Rather, it is likely that functionally, CD151 (and, possibly, other tetraspanins) are linked to cPKC indirectly. Indeed, we show here for the first time that the removal of CD151 in breast cancer cells decreases the abundance of particular species of DAG. The importance of this observation is that it is not a general change in all DAG rather specific changes in individual

molecular species. We have previously discussed the differential regulation of signaling by distinct DAG species (Wakelam, 1998). Furthermore, our data indicate that the effect of CD151 on the production of DAGs (at least, some of the DAG species) may be linked with redistribution of PLCγ. Thus we propose that CD151 affects the activity of PKC by regulating either biosynthetic or catabolic pathways linked to the generation of DAGs. Further investigation will be necessary to pinpoint how these pathways are directly targeted by the tetraspanin.

In summary, we discovered a novel mechanistic link between the CD151-complex and FGFR2. The data demonstrate that, in addition to their well established role as post-translational regulators of protein expression, tetraspanins are also involved in regulation of protein expression at the post-transcriptional level. Future investigation into molecular pathways responsible for tetraspanin/integrin-dependent regulation of FGFR2 expression will further define functional interdependence of tetraspanin microdomains and FGFR2 in the context of breast cancer.

MATERIALS AND METHODS

Cell lines, antibodies and reagents. HB2/CD151(+), HB2/CD151(-), HB2/α3(-), HB2/α6(-), SKBR3/CD151(+), SKBR3/CD151(-), MCF7/CD151(+) and MCF7/CD151(-) cells were described previously (Baldwin et al., 2008; Novitskaya et al., 2010; Novitskaya et al., 2014). HB2/CD151rec cell line was established after transfections of HB2/CD151(−) cells with the construct encoding shRNA-resistant form of CD151 (Novitskaya et al., 2014). Antibodies to FGFR1 (sc-121), FGFR2 (sc-122), FGFR4 (sc-124), Src (sc-18), TIA-1 (sc-1751) were from Santa Cruz. Antibodies to β-actin (A5316) were purchased from Sigma-Aldrich; all phospho-specific antibodies (phospho (Ser) PKC substrate (#2261), phospho Src Family (Tyr 416) (#2113), phospho p38 (T180/Y182) (#9215) and phospho-PLCgamma1(Y783)(#2821)) and antibodies to EDC4/Ge-1 (#2548), PatL1 (#14288), TIAR (#8509), hnRNP C1/C2 (#12392) and HuR/ELAVL-1 (#12582) were from Cell Signaling Technologies. All growth factors were purchased from Peprotech; laminin-rich extracellular matrix (lrECM) - Matrigel, was from BD Bioscience.

Culturing of cells in 3D lrECM. Culturing cells in 3D-lrECM was performed

as previously described (Sadej et al., 2009). Briefly, 1.5 × 10^3 cells were resuspended in 40μl of growth factor reduced lrECM:DMEM (1:1). Solidified lr-ECM drops were covered with medium containing 2% FBS and supplemented, when needed, with FGFs (50 ng/ml). For morphological analyses in 3D culture experiments, pictures were taken using a Zeiss Primovert microscope coupled with camera (AxioCam ERc 5s, Zeiss). Cell proliferation in 3D was measured by WST1 reagent (tetrazolium salts) according to the manufacturer's protocol (Roche). Experiments were performed in duplicates, and at least three independent experiments were done for each experimental condition. Comparative data were analysed with the unpaired Student's *t*-test using the Statistica 7.1 software. Differences for which $p<0.05$ were considered as statistically significant.

Western blotting. Cells grown to 80-90% confluence were lysed in Laemmli buffer supplemented with 2 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 5 mM EGTA, 1 mM EDTA, 2 mM $Na_4P_2O_7$, 5 mM NaF and 5 mM Na_3VO_4. Samples containing equal amounts of protein per lane were loaded and resolved on 10% SDS-PAGE and then transferred onto nitrocellulose membrane. The membranes were incubated for 1 hour in 5% skimmed milk and probed with specific antibodies overnight. The infrared-tagged secondary antibodies were used to visualise the signals, and the images were capture and quantified using a LI-COR Odyssey scanning system.

siRNA-based knockdown. Cells were transfected with control or gene-targeting siRNAs (20 nM) using Lipofectamine®RNAiMAX transfection reagent (ThermoFisher). The expression level of FGFR2 was assessed by western blotting 72 hours after transfection. For immunofluorescence experiments, cells were plated on coverslips the day before transfection and analysed 72 hours after transfection. All siRNA were purchased from Qiagen in the Flexitube format; target sequences are shown in Supplementary Table1

Immunofluorescence staining. Cells grown on glass coverslips were fixed with 4% paraformaldehyde and permeabilised using 0.1% Triton X-100. Staining with rabbit anti-EDC4 Ab (diluted at 1:200) was carried out according to the manufacture's recommendations. P-bodies were quantified in 50-100 cells in 2-3 independent experiments. For staining using anti- PLCγ1 antibody

(diluted at 1:100, Cell Signaling Technology, #2282) cells were permeabilised for 1h in 1%Brij98.

Real time qPCR. Total RNA was isolated from HB2 cells using the RNAeasy kit according to the manufacturer's instructions (Qiagen, Crawley, United Kingdom). The cDNAs were synthesized from RNA (1 μg) by MultiScribe™ MuLV reverse-transcriptase (Life Technology, Paisley, United Kingdom). Real time PCR was carried out using either commercial TaqMan or custom designed SYBR Green primers (sequences are available upon request). PCR conditions were as follows: (a) 95°C for 10min; (b) 40 cycles of 95°C for 15 s and 60°C for 1 min. At least three separate PCR reactions for each gene were performed. The real-time amplification data were analysed using REST software (Qiagen, Crawley, United Kingdom) and gene expression levels were normalized relative to the control GAPDH gene for TaqMan assay or β-Actin for SYBR green assay.

Diacylglycerol analysis by mass spectrometry. Frozen cell pellet lipids were extracted using the Folch method with Chloroform/Methanol/Water (2:1:1 ratio) and resuspended in Chloroform/Methanol (1:1) prior to injection into a Shimadzu Prominence 20-AD system (Shimadzu, Kyoto, Japan). Chromatographic separation was achieved upon a Waters Acquiity UPLC C_4 (100 x 1 mm, 1.7 µm particle size) column (Milford, MA, U.S.A.). The column was kept a 45 °C and 7 µl of samples were eluted using a mobile phase composed of solvent A: water and B: acetronitile each containing 0.025 % formic acid. The gradient started at 45 % B (5 min), with an increase to 90 % B for 5 min, 100 % B was reached after an additional 10 minutes and held for 7 min before reequilibration at 45% B for 5 minutes. The flow rate was maintained constant at 100 µL/min. Accurate mass (with an error below 5 ppm) was acquired on an Orbitrap Elite mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Source parameters for positive polarity were: capillary temperature 275 °C; source heater temperature 200 °C; sheath gas 10 AU; aux gas 5 AU; sweep gas 5 AU. Source voltage was 3.8 kV. Full scan spectra in the range of *m/z* 150 to 1,000 were acquired at a target resolution of 240,000 (FWHM at *m/z* 400). Data analysis was performed using Lipid Data Analyzer (2.6.0_2) software (Hartler et al., 2017).

Patient Selection and Samples are described in Supplementary Table2. Specimens of primary invasive ductal carcinoma from women treated in the Oncology Department of the Copernicus Memorial Hospital in Lodz, Poland between 2003 and 2010 were obtained according to the local ethical regulations (License No. RNN/174/11KE, Medical University of Lodz 2011). This was an archival material and no consent was (then) required.

Immunohistochemistry. Serial 5μm paraffin sections of archival formalin - fixed blocks were processed for immunohistochemistry for FGFR2 (rabbit anti-human; 1:100, Santa Cruz, UK) and CD151 (mouse anti-human; 1:100; Novocastra, UK) using routine protocols described previously (Novitskaya et al., 2014). As a negative control for immunostaining, primary antibodies were replaced by nonimmune sera. Scoring immunoreactivity for FGFR2 was carried out separately for cytoplasmic/membranous and nuclear FGFR2 expression. Cytoplasmic/membranous expression of FGFR2 was considered : i) 0/negative – if no reactivity, ii) 1+/positive – if weak to moderate membranous and/ or cytoplasmic staining in <10% of tumour cells; iii) 2+/positive – if moderate membranous and/or cytoplasmic staining in ≥10% of tumour cells; iv) 3+/positive – if strong membranous and/or cytoplasmic staining in ≥ 10% of the tumour cells. Assessment of nuclear immunoreactivity was based on Qiuck score (Detre et al., 1995). Scoring of immunoreactivity for CD151 was described previously (Novitskaya et al., 2014). Immunohistochemical staining was assessed independently by two observers (HR and RK) blinded to the clinicopathological data.

Statistical analysis. Overall survival was calculated from the date of surgery to the date of death or the last follow-up, as recommended by the Kaplan-Meier method. Differences in survival distributions were compared using log-rank test. Data for patients who died from other causes than breast cancer were censored at the time of death. Univariate and multivariate analyses of overall survival were performed using the Cox's proportional hazards regression model. Pearson's test or Fisher's exact test were used to assess the associations between expression of FGFR2 and CD151 alone and their coexpression and clinico-pathological variables. Kendall's tau rank correlation test was used to study correlation between levels of FGFR2 and

CD151 expression in cancer tissue. The results were considered statistically significant when two-sided p was less than 0.05. The analyses were performed using the StatsDirect (StatsDirect Ltd, Altrincham, UK) and Statistica 9.1 (StatSoft Inc. Tulsa, OK, USA) software.

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Competing interests: No competing interests declared

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Tables

Table 1. Relationship between clinicopathological features and expression of i) FGFR2 alone and ii) in association with CD151.

Feature	**FGFR2+** (n=65) vs. rest (n=101)	**FGFR2+/CD151-** (n=38) vs. rest (n=128)	**FGFR2-/CD151+** (n=55) vs. rest (n=111)	**FGFR2+/CD151+** (n=27) vs. rest (n=139)	**FGFR2-/CD151-** (n=50) vs. rest (n=116)
Tumour size	0.131	0.344	0.164	0.487	0.839
Nodal Status	0.724	0.933	**0.037**	0.574	**0.013**
Stage	0.510	0.366	0.114	>0.999	0.369
Grade	0.095	**0.012**	0.102	0.510	0.895
HER2	0.197	0.771	0.281	0.171	**0.014**
ER/PR	**0.040**	0.221	0.066	0.185	0.736
TN	0.293	0.595	**0.039**	0.437	0.333

Table 2. Univariate analysis of prognostic factors.

Factor	Hazard Ratio	95%CI	P value
FGFR2	1.25	0.752.07	0.383
CD151	1.80	1.083.01	**0.023**
FGFR2+/CD151-	0.95	0.521.73	0.87
FGFR2-/CD151+	1.45	0.872.44	0.16
FGFR2+/CD151+	1.54	0.842.84	0.17
FGFR2-/CD151-	0.47	0.240.91	**0.024**
Tumour size (T1 vs T24)	1.77	1.302.40	**<0.001**
Nodal status	3.01	1.785.10	**<0.001**
Stage (I vs II vs III)	1.81	1.272.57	**<0.001**
ER/PR	0.53	0.330.87	**0.011**
HER2	2.07	1.183.64	**0.012**
Grade (G12 vs G3)	1.24	0.762.01	0.383

Figures

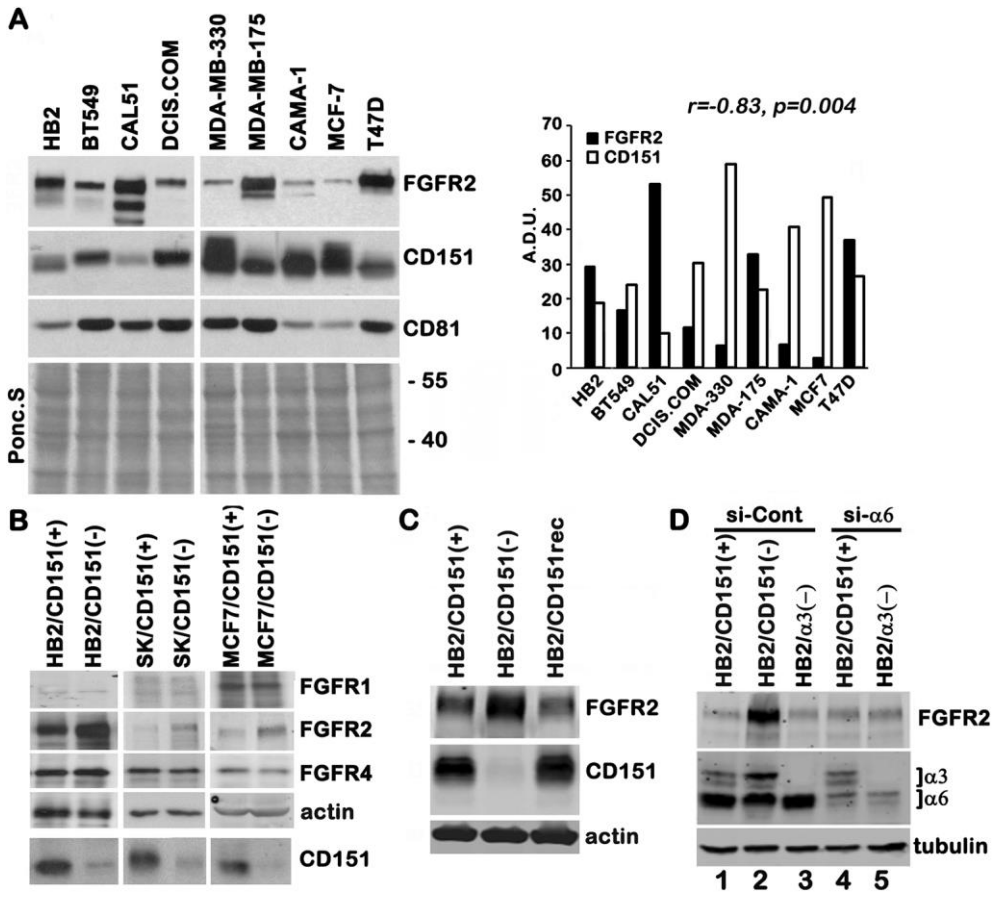


Figure 1. CD151 regulates expression of FGFR2 gene. A) Inverse correlation between CD151 and FGFR2 expression in a panel of breast cancer cell lines. Protein lysates (20 μg) were resolved in SDS-PAGE and expression levels of CD151, CD81 and FGFR2 were analysed by Western blotting. Membranes were stained with Ponceau S to control for the amounts of loading material. Right panel displays arbitrary density units for FGFR2 and CD151 signals. Pearson's correlation coefficient was calculated using Excel 2010. **B)** Knockdown of CD151 in mammary epithelial cells results in a specific increase of FGFR2 expression without affecting other FGF receptors. Expression of FGF receptors was analysed as described in A. **C)** Stable re-expression of shRNA-resistant form of CD151 in CD151-depleted cells (HB2/CD151rec) reversed FGFR2 expression in HB2 cells. **D)** Silencing of α3 and α6 integrins does not affect the expression level of FGFR2. Expression

of FGFR2 was analysed in HB2 cells depleted of CD151 (lane2), $\alpha 3$ integrin subunit (lane 3), $\alpha 6$ integrin subunit (lane 4) and $(\alpha 3+\alpha 6)$ integrin subunits (lane 5). HB2/CD151(+) cells were used as a control. Middle panel shows expression levels of $\alpha 3$ and $\alpha 6$ integrin subunits.

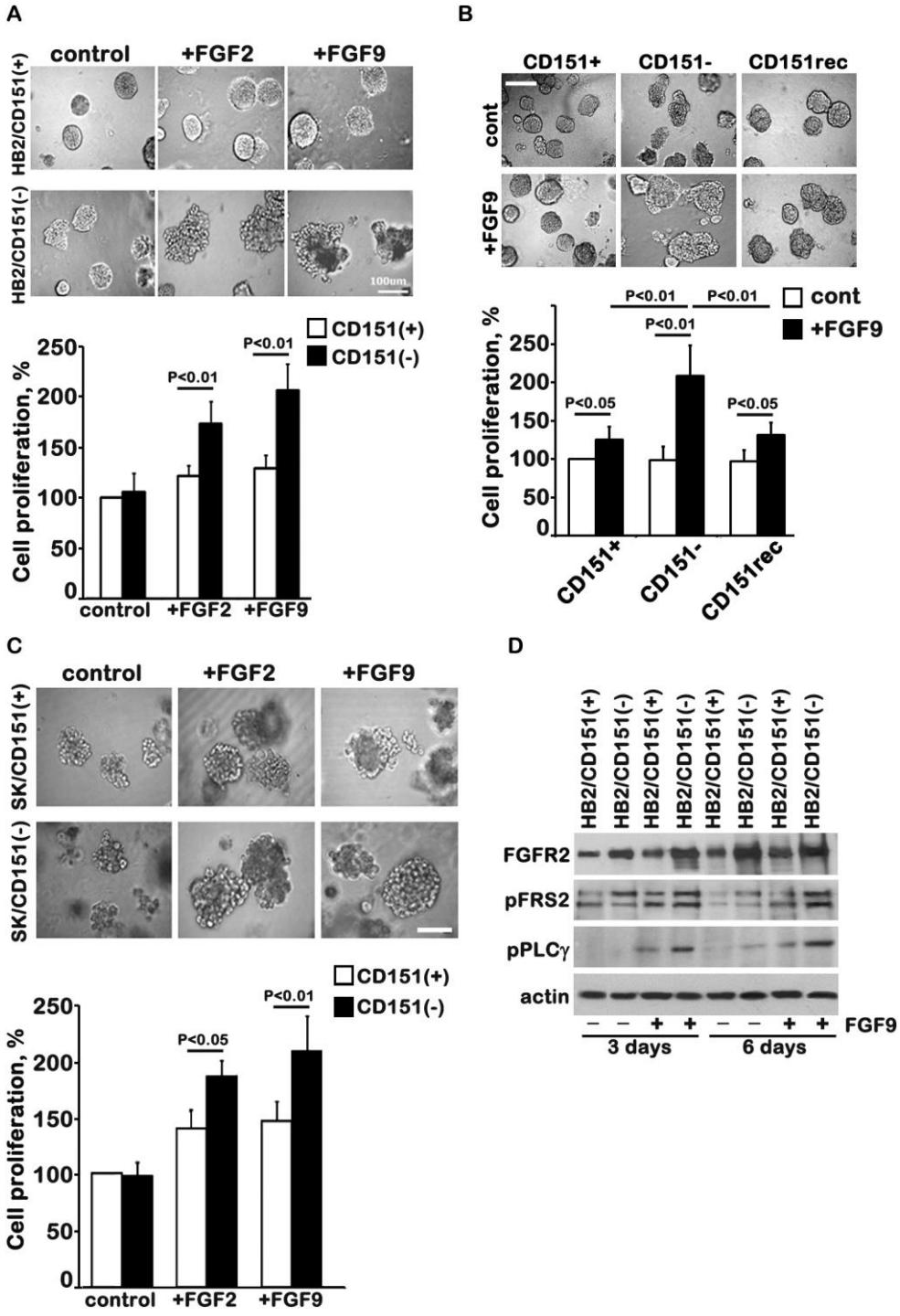


Figure 2. CD151 regulates cellular responses to FGFs. HB2 (**A,B**) and SKBR3 (**C**) CD151(+) vs. CD151(-) cells were embedded in 3D lrECM and grown for 8 days in a presence of FGF2 and FGF9 (50 ng/ml). Representative images are shown. Proliferation was measured using WST1 reagent as described in Material and Methods and shown as histograms in which bars represent averaged results (mean) of three independent experiments. Error

bars represent SD, p values were calculated using two-tailed t-test and are indicated on graphs. Proliferation is presented as percentage of the control, CD151-positive cells grown under standard culturing conditions. **D)** Activation of FGFR2-dependent signaling pathways in cells grown under standard culturing conditions was analysed by Western blotting. Shown representative results of one of three independent experiments.

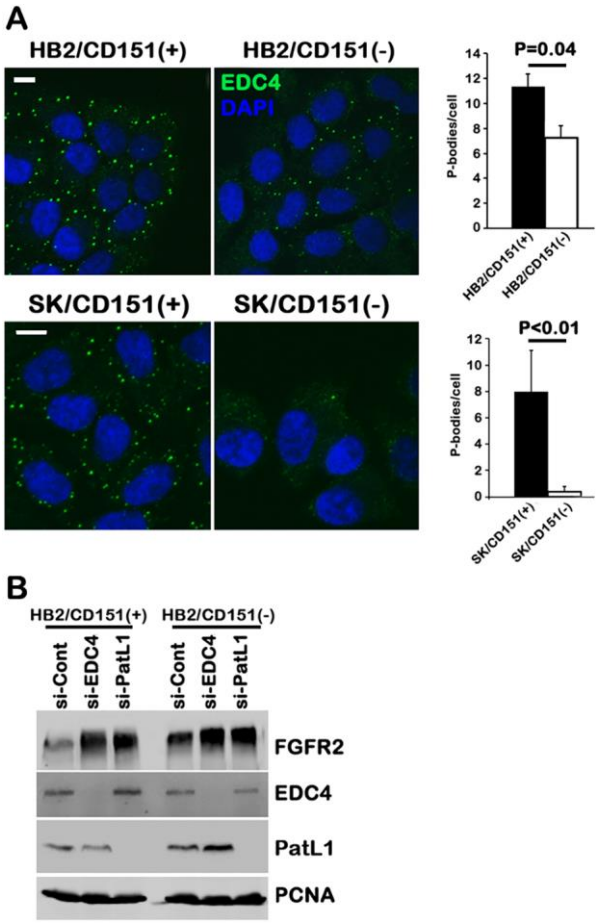


Figure 3. The effect of CD151 depletion on the formation of P-bodies, and the role of P-bodies in the expression of FGFR2. A) The number of P-bodies is decreased in CD151-depleted cells. Cells were grown under standard culturing conditions for 48h before staining with the anti-EDC4 antibody to reveal P-bodies. Shown, representative images. Numbers of P-bodies was quantified in at least 70-100 cells. Scale bar, 10 μm. On the left panel bars represent averaged results (mean) of number of P-bodies per cell. Error bars represent SD, p value was calculated using two-tailed t-test. **B)** Knockdown of the key components of P-bodies (EDC4 and PatL1) increases FGFR2 expression. Cells were depleted of EDC4 and PatL1 using specific siRNAs. The expression of FGFR2 was assessed by Western blotting 72 hours after transfection. Shown results of one of two independent experiments

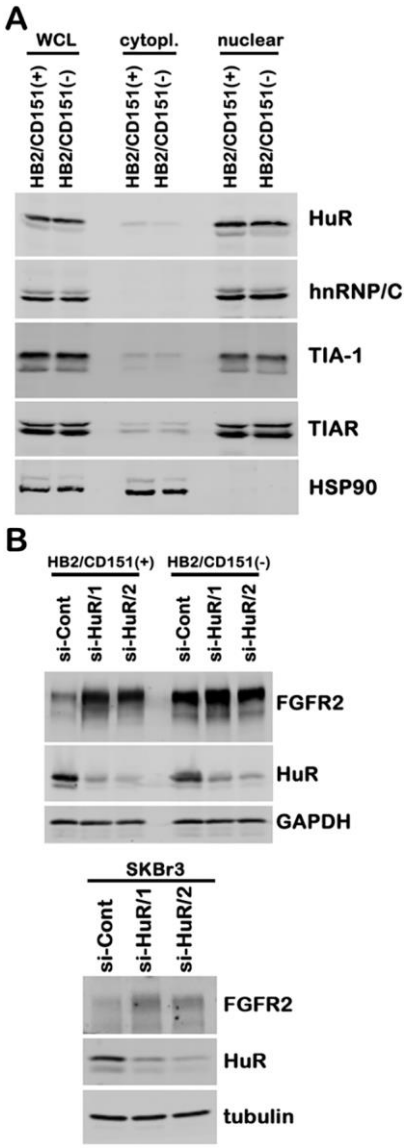


Figure 4. The role of HuR/ELAVL-1 in CD151-dependent regulation of FGFR2 expression. **A**) Expression levels and distribution of mRNA-binding proteins in HB2 cells was analysed by Western blotting using specific antibodies. WCL – whole cell lysates; cytopl. and nuclear – cytoplasmic and nuclear fractions of the proteins, respectively. Note, HSP90 is only found in the cytoplasmic fractions. **B**) The effect of HuR/ELAVL-1 siRNA knockdown on the FGFR2 expression in HB2 and SKBr3 cells. The expression of FGFR2 was assessed by Western blotting 72 hours after transfection. Shown results of one of the two independent experiments.

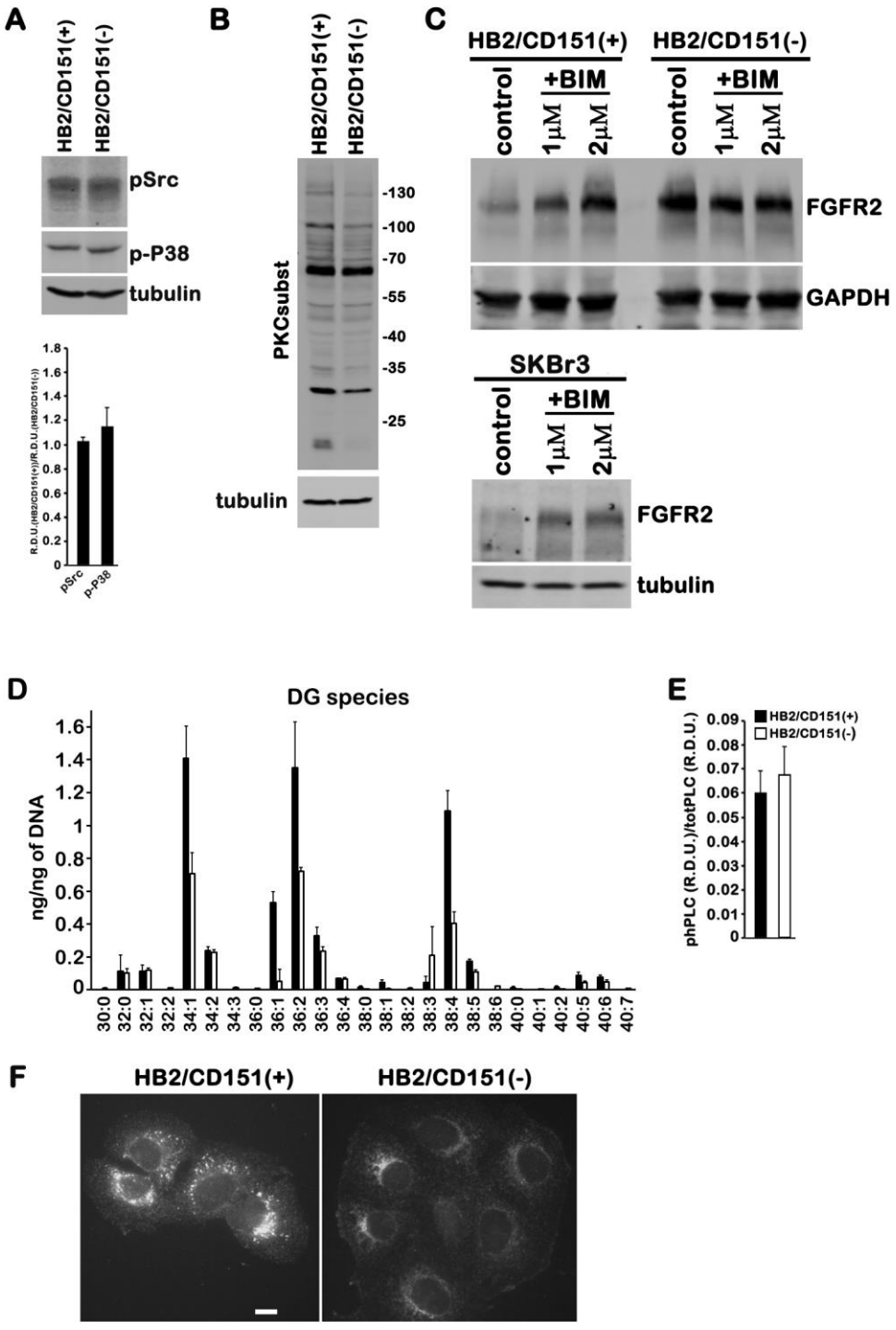


Figure 5. The role of PKC in CD151-dependent regulation of the FGFR2 expression. A) Activation of p38 and Src in cells grown under standard culturing conditions was analysed by Western blotting using appropriate phospho specific Abs. Histogram shows compilation of densitometric measurements of three independent experiments. R.D.U. – relative density units. **B)** Activation of PKC in cells grown under standard culturing conditions

was analysed by Western blotting using phospho-(Ser) PKC substrate antibody. **C)** Cells were grown under standard culturing conditions in the presence of BIM-I for 72h and the expression of FGFR2 was assessed by Western blotting. Shown a representative image of three independent experiments. **D)** Determination of diacylglycerol species**.** The concentrations of individual DAG molecular species were determined by mass spectrometry in HB2/CD151(+) (closed bars) and HB2/CD151(-) cells (open bars) and normalized to cellular DNA content. n=4 in each case. **E)** Activation of PLCγ was assessed by western blotting using anti-phPLCγ (Tyr783) Ab in 3 independently prepared cellular lysates. Shown means of ratios signal intensities of phPLCγ/total-PLCγ. **F)** Distribution of PLCγ in HB2/CD151(+) and HB2/CD151(-) cells. Cells were grown under standard culturing conditions for 48h before staining with the anti- PLCγ antibody. Shown, representative images. Scale bar, 10 μm.

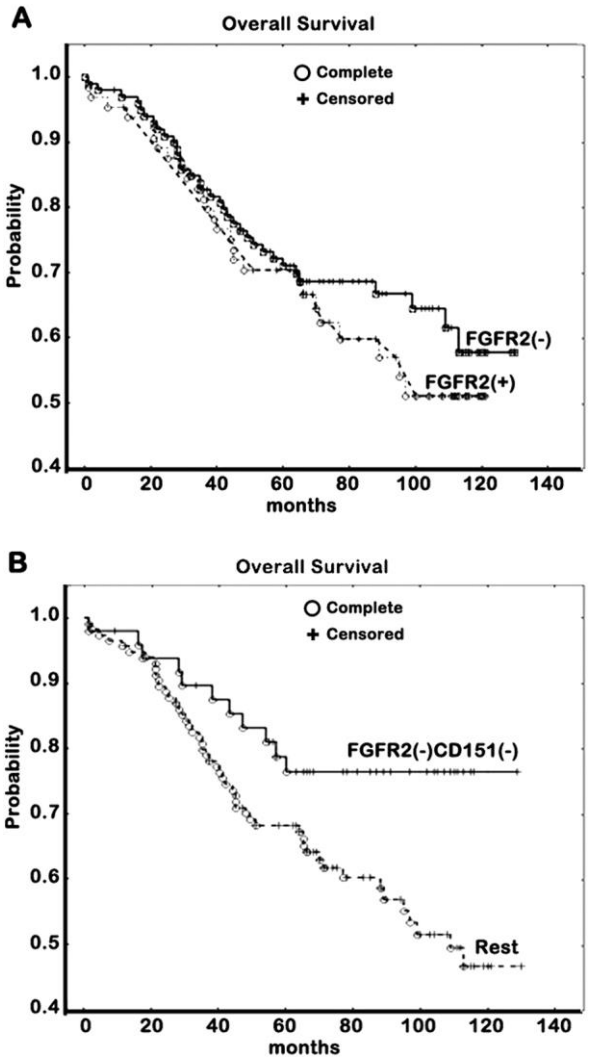
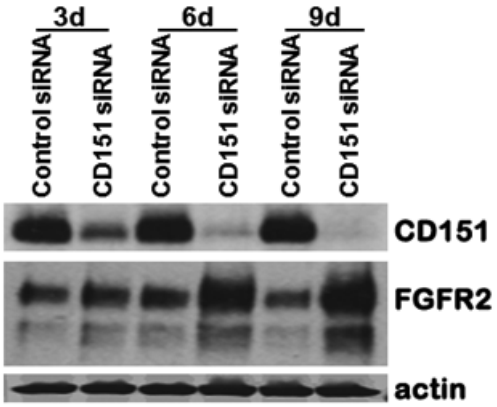


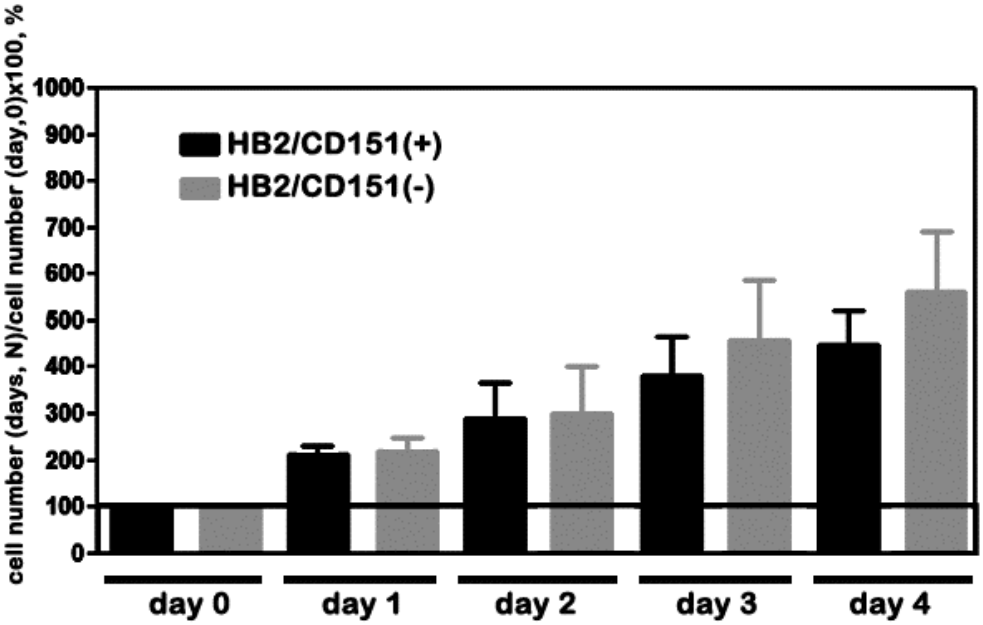
Figure 6. Kaplan-Meier curves of overall survival for patients with breast cancer. A. Patients expressing cytoplasmic/membranous FGFR2 compared with FGFR2-negative patients. **B.** Patients negative for both FGFR2 and CD151 (FGFR2(-)CD151(-)) compared with the rest of the cohort.

Supplementary Fig.1



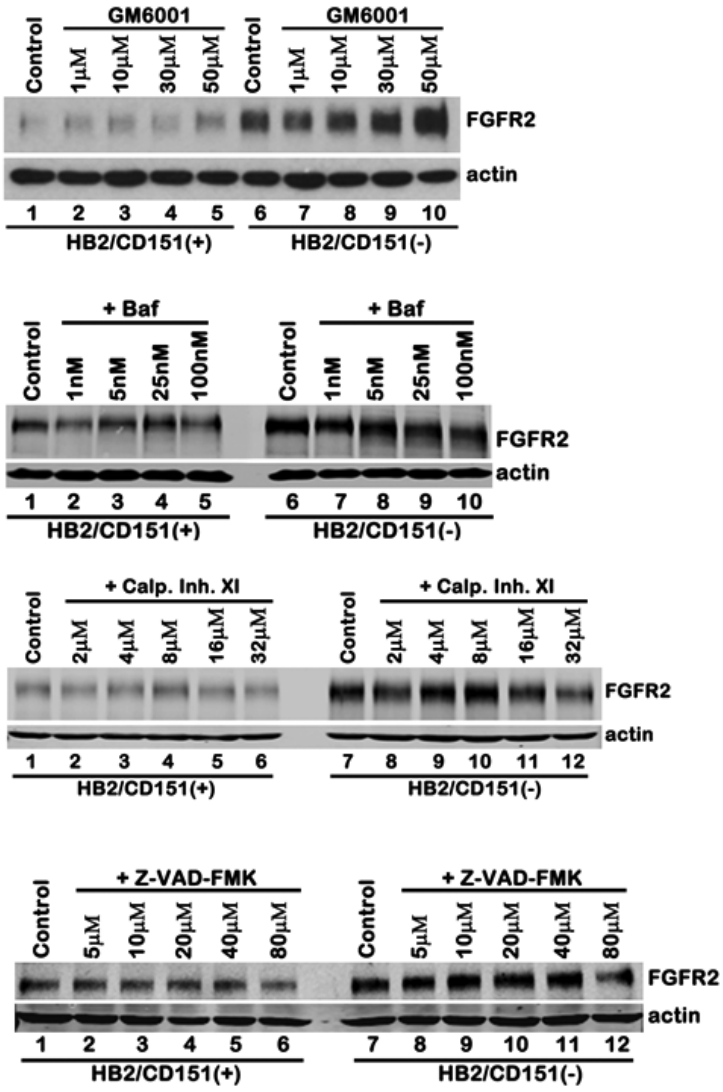
Transient knockdown of CD151 in HB2 cells resulted in the increased level of FGFR2. Note, the correlation between the degree of CD151 knockdown and the increase in the expression level of FGFR2.

Supplementary Fig.2



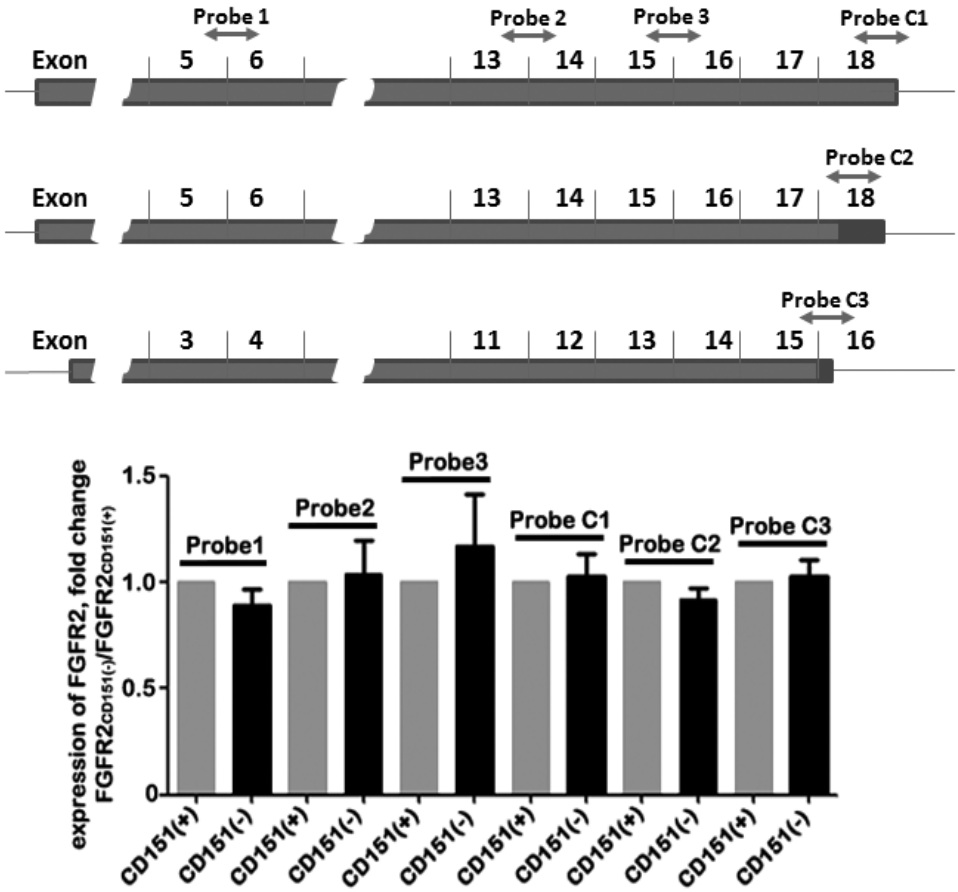
Knockdown of CD151 does not affect proliferative responses of HB2 cells to FGF2. Cells were serum starved and subsequently incubated in serum-free growth media containing FGF2 (50ng/ml) for indicated time intervals. Number of cells on a given day was counted, and ratio to the number of cells plated ("day 0") was calculated and multiplied by 100. Cells were plated in triplicated for each of the time points.

Supplementary Fig.3



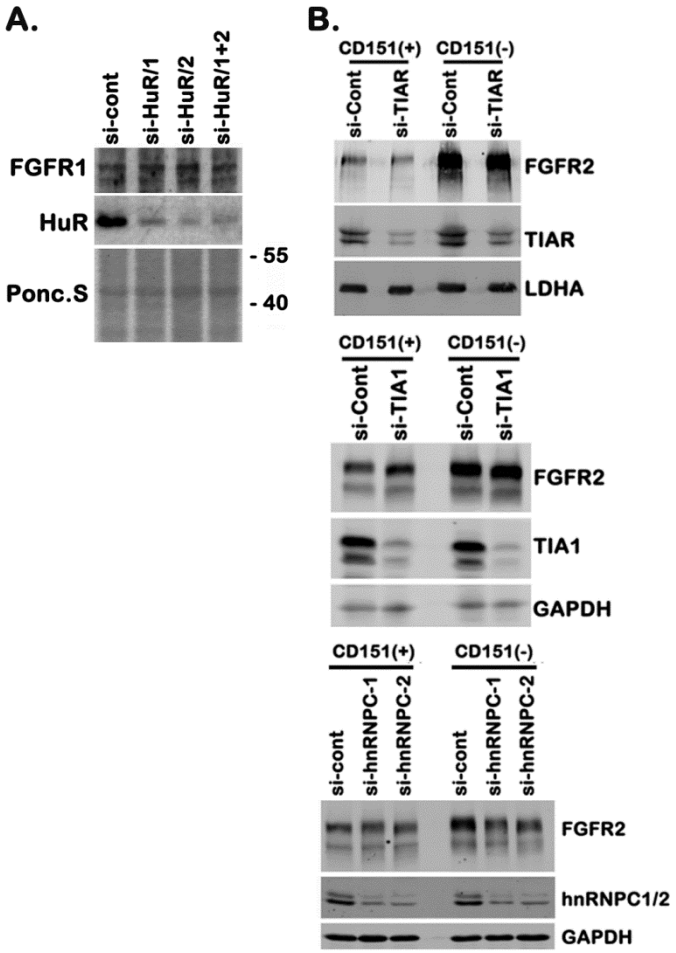
The effect of proteolytic inhibitors on the expression levels of FGFR2 in HB2 cells. Cells were incubated with indicated concentrations of the inhibitors for 16 hours and the expression of FGFR2 was analysed by Western blotting.

Supplementary Fig.4



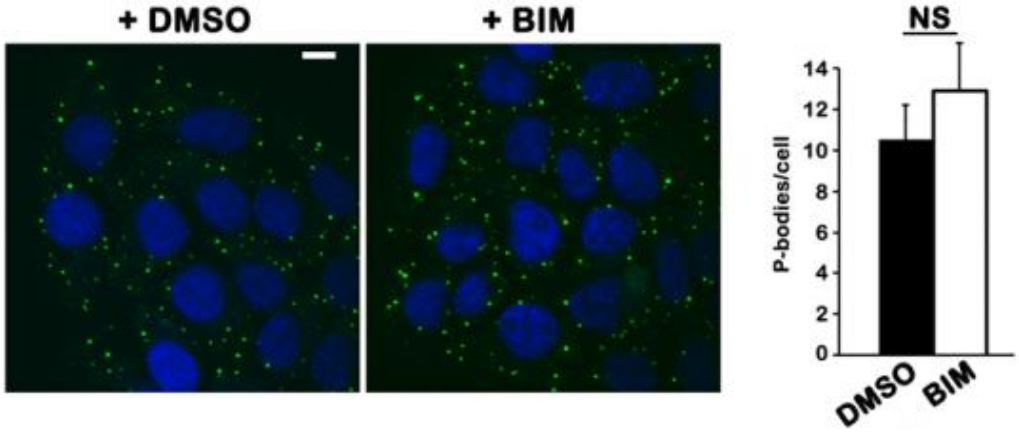
Analysis of the mRNA FGFR2 expression in HB2 cells by RT-qPCR. Top diagram shown positions of the amplified regions in the mRNA FGFR2.

Supplementary Fig.5



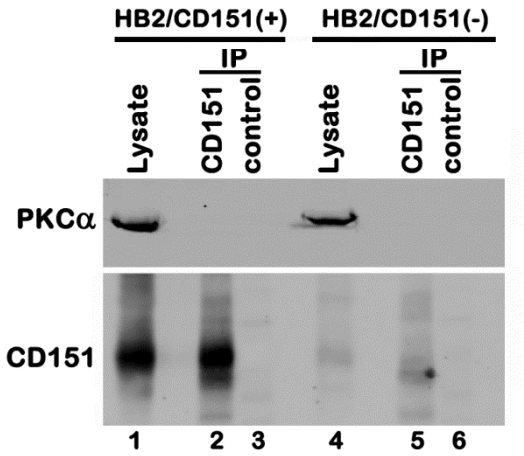
A. Knockdown of HuR/ELAVL-1 does not affect expression of FGFR2. **B.** Knockdown of various RNA binding proteins has no or minimal effect on the FGFR2 expression in HB2/CD151(+) and HB2/CD151(-) cells, “CD151(+)” and “CD151(-)” respectively. The expression of FGFR2 was assessed by Western blotting 72 hours after transfection.

Supplementary Fig.6



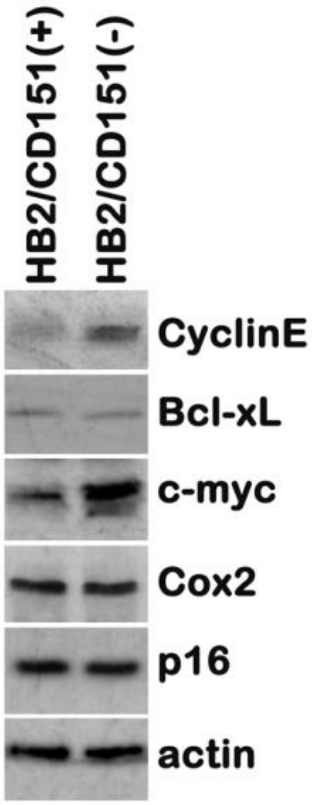
Treatment of HB2/CD151(+) cells with a PKC inhibitor has no effect on the assembly of P-bodies. Cells were grown under standard culturing conditions in media supplemented with BIM I (2μM) for 72 hours with daily media change. P-bodies were visualised with anti-EDC4 Ab. Numbers of P-bodies was quantified in at least 30-50 cells. Scale bar, 10 μm.

Supplementary Fig. 7



Cells grown under standard culturing conditions were lysed in 1%Brij98 and CD151-containing complexes were precipitated using mouse anti-CD151 mAb (5C11). The complexes were resolved in SDS-PAGE and the presence of PKCα in the complex was examined using rabbit anti-PKCα Ab. Mouse anti-GFP mAb were used as isotype-specific negative control.

Supplementary Fig. 8



The effect of CD151 knockdown on the expression of cellular proteins that were identified as HuR/ELAVL-1 targets.

Supplementary Table 1

gene	siRNA sequence
PatL1	GGACCUUUCUGAACGAGCA
PatL1	CGUCGACUCUUGCAUCAGA
TIA-1	CGCUCCAAAGAGUACAUAU
TIA-1	GAUAUUCAUUUGUUCGGUU
TIAL	GGAUUUGGAGUAGAUCAAU
TIAL	GUAAACCACCUGCACCUAA
EDC4	GGUGUCUGCACGAGUGGAA
EDC4	GGAGAUGAUAGCUCCACCU
hnRNPC	AACGUCAGCGUGUAUCAGGAA
hnRNPC	UUGGUGAUACCUCAUCCUUGA
ELAVL1/HuR	AAGUAGCAGGACACAGCUUGG
ELAVL1/HuR	ACCAGUUUCAAUGGUCAUAAA
α6 integrin subunit	GGUCGUGACAUGUGCUCAC
α6 integrin subunit	CAAGACAGCUCAUAUUGAUUU
CD151	CAUGUGGCACCGUUUGCCU

Supplementary Table 2.

Patient characteristics.

Number of patients	166
Age (years)	
< 50	52
≥ 50	114
Disease stage	
I	39
II	84
III	43
T status	
T1	55
T2	103
T3	1
T4	7
Grade	
1-2	95
3	71
Nodal status	
Negative	82
Positive	84
Steroid receptor status	
Negative	73
Positive	93
HER2 status	
Negative	138
Positive	28